VERIFICATION OF TRANSLATION

Patent Application No. 2002-244280

I, Hidejiro TANIGAWA of 4-13-802, Fujimi 2-chome, Chiyoda-ku, Tokyo 102-0071, Japan, am the translator of the documents attached and I state that the following is a true translation to the best of my knowledge and belief of Japanese Patent Application No. 2003-373779.

DATED this 27th day of May, 2009

Signature of translator Hidejiro Tanigawa

Hidejiro TANIGAWA

[TITLE OF DOCUMENT] REQUEST FOR PATENT

[DOCKET NUMBER] P0837

[TO] Director General of Patent Office

[INTERNATIONAL PATENT CLASSIFICATION] C12N 5/12 G01N 33/53

[INVENTOR]

[ADDRESS OR DOMICILE] c/o FUJIREBIO INC., 62-5, Nihonbashi-Hamacho 2-chome, Chuo-ku, Tokyo 103-0007 Japan

[NAME] UCHIDA Yoshiaki

[INVENTOR]

[ADDRESS OR DOMICILE] c/o FUJIREBIO INC., 62-5, Nihonbashi-Hamacho 2-chome, Chuo-ku, Tokyo 103-0007 Japan

[NAME] FUJII Nobuyuki

[INVENTOR]

[ADDRESS OR DOMICILE] c/o FUJIREBIO INC., 62-5, Nihonbashi-Hamacho 2-chome, Chuo-ku, Tokyo 103-0007 Japan

[NAME] KURANO Yoshihiro

[INVENTOR]

[ADDRESS OR DOMICILE] c/o FUJIREBIO INC., 62-5, Nihonbashi-Hamacho 2-chome, Chuo-ku, Tokyo 103-0007 Japan

[NAME] OKADA Masahisa

[APPLICANT FOR PATENT]

[IDENTIFICATION NUMBER] 000237204

[NAME] FUJIREBIO INC

[REPRESENTATIVE] SUZUKI Hiromasa

[INDICATION OF FEE]

[ADVANCED PAYMENT BOOK NUMBER] 011556

[AMOUNT] 21000

[LIST OF DOCUMENTS SUBMITTED]

[TITLE OF DOCUMENT] Claims 1

[TITLE OF DOCUMENT] Specification 1

[TITLE OF DOCUMENT] Drawing 1

[TITLE OF DOCUMENT] Abstract 1

【TITLE OF DOCUMENT】 CLAIMS

【Claim 1】

An anti-SARS virus monoclonal antibody against nucleoprotein of a corona virus which causes severe acute respiratory syndrome (SARS).

【Claim 2】

The anti-SARS virus monoclonal antibody according to claim 1, which monoclonal antibody is prepared by using as an immunogen the nucleoprotein of said coronavirus, said nucleoprotein being expressed by a vector in which a nucleotide sequence shown in SEQUENCE LISTING 1 is incorporated.

【Claim 3】

The anti-SARS virus monoclonal antibody according to claim 2, which monoclonal antibody is produced by hybridoma rSN-18 having an Accession No. FERM P-19572, hybridoma rSN-122 having an Accession No. FERM P-19573 or hybridoma rSN-150 having an Accession No. FERM P-19574.

【Claim 4】

A hybridoma producing said monoclonal antibody according to any one of claims 1 to 3, which hybridoma is obtained by fusing an anti-SARS virus monoclonal antibody-producing cell and a tumor cell.

【Claim 5】

A reagent for immunoassay of SARS-causing coronavirus, comprising said monoclonal antibody according to any one of claims 1 to 3 as the immobilized antibody and/or labeled antibody.

【TITLE OF DOCUMENT】 SPECIFICATION

【TITLE OF THE INVENTION】 Anti-SARS Virus Antibody, Hybridoma Producing the Antibody and Immunoassay Reagent Using the Antibody

【Technical Field】

【0001】

The present invention relates to a monoclonal antibody against the nucleoprotein of the severe acute respiratory syndrome (SARS)-causing coronavirus (hereinafter referred to as "SARS virus"), hybridoma which produces the monoclonal antibody, and to an immunoassay reagent for SARS virus, which uses the monoclonal antibody as the immobilized antibody and/or labeled antibody.

【Background Art】

【0002】

From 2002 to 2003, patients suffering from severe pneumonia were reported worldwide, and a number of death were reported in addition to the infected patients. The virus isolated from the patients was named SARS virus, and the virus was confirmed to be a new type of coronavirus. The whole genome has been sequenced by Michael Smith Genome Sciences Centre in British Columbia, Canada (Non-patent Literature 1).

After incubation period of 2 to 7 days from the infection by SARS virus, the SARS virus causes high fever higher than 38°C, coughs, headache, dyspnea and so on. Since the symptoms of SARS are similar to those of influenza, diagnosis whether the infection is by SARS or not at an early stage is demanded. Reported diagnoses of infection by SARS virus include the following:

1) Measurement of Antibody by ELISA: Antibodies (IgM/IgA) in sera of SARS patients may be detected after about 20 days from the manifestation of clinical symptoms.

2) Immunofluorescence Method: Immunofluorescence method using VERO

cells infected with SARS virus (detecting IgM). Antibody in serum may be detected after about 10 days from the onset.

3) PCR Method: SARS virus gene products from various specimens such as blood, feces and respiratory secretions are amplified and detected.

4) Cell Culture Method: Virus in a specimen from a SARS patient is infected to culture cells such as VERO cells and then detected.

【0003】

【Non-patent Literature 1】 Science; 2003 May 30;300(5624):1394-9

【Disclosure of the Invention】

【Problems Which the Invention Tries to Solve】

【0004】

Among the known methods for confirming infection by SARS virus, with the antibody test methods, the infection can be detected only after 10 days from the infection, and the highly reliable immunofluorescence method is complicated. As for the PCR method, since it is necessary to isolate and amplify a SARS-related gene, the method requires a special amplification apparatus and measurement apparatus, and is not a simple measurement method. As for the cell culture method, it is difficult to process a number of specimens, and infection by SARS virus cannot be confirmed only by this method, even though the infection by coronavirus may be confirmed.

In view of the above-described circumstances, an object of the present invention is to provide a monoclonal antibody which specifically recognizes SARS virus, and to provide an immunoassay reagent for detecting SARS virus which utilizes the monoclonal antibody.

【Means to Solve the Problems】

【0005】

The present inventors intensively studied for obtaining an anti-SARS virus

monoclonal antibody having specificity to SARS virus and having a high affinity to obtain the desired monoclonal antibody by obtaining a nucleoprotein gene of SARS virus from synthetic nucleotide synthesized by utilizing PCR; preparing a transformant using the obtained gene in accordance with gene manipulation techniques; and using, as an immunogen, the nucleoprotein of SARS virus obtained from the transformant. Further, the present inventors were able to develop an immunoassay reagent using the monoclonal antibody.

【0006】

The monoclonal antibody according to the present invention is a monoclonal antibody which specifically recognizes the nucleoprotein of SARS virus. Further, the present invention is a hybridoma which produces said monoclonal antibody. Further, the present invention is an immunoassay reagent for the nucleoprotein of SARS virus, which is characterized by measuring a nucleoprotein of SARS virus by reacting said monoclonal antibody with a sample and measuring formed immune complex.

【Effect of the Invention】

【0007】

Since the monoclonal antibody according to the present invention has a high specificity and high reactivity to the nucleoprotein of SARS virus, the monoclonal antibody may be used for highly sensitive immunoassay of SARS virus. The hybridoma according to the present invention can provide a monoclonal antibody which specifically recognizes SARS virus. Further, the immunoassay reagent utilizing the monoclonal antibody according to the present invention may detect only the samples from SARS patients by simple operations.

【Best Mode for Carrying out the Invention】

【0008】

The present invention will now be described in detail. The monoclonal

antibody according to the present invention is a monoclonal antibody which specifically reacts with the nucleoprotein of SARS virus. To obtain the monoclonal antibody according to the present invention, the nucleoprotein, preferably the nucleoprotein produced in accordance with gene manipulation, may be used as an immunogen. As the recombinant antigen, the polypeptide substantially containing the amino acid sequence shown in SEQ ID NO:1 in SEQUENCE LISTING may be used.

【0009】

In the present invention, the monoclonal antibody having high specificity to the nucleoprotein of SARS virus may be obtained by using as an immunogen the above-described polypeptide substantially containing the amino acid sequence shown in SEQ ID NO:1 in SEQUENCE LISTING.

【0010】

As the nucleoprotein of SARS virus used as the above-described immunogen, any polypeptides may be available as long as the polypeptides substantially contain the amino acid sequence shown in SEQ ID NO:1 in SEQUENCE LISTING, and whole region of the nucleoprotein of SARS virus may also be used as an immunogen. The nucleoprotein may not be necessarily highly purified, and crudely purified nucleoprotein may also be used as an immunogen. "Polypeptides substantially containing the amino acid sequence shown in SEQ ID NO:1 in SEQUENCE LISTING" means that the polypeptides include those containing the same amino acid sequence shown in SEQ ID NO:1 except that one or several amino acids in the amino acid sequence shown in SEQ ID NO:1 are deleted, substituted and/or added to the extent that the function or three-dimensional structure of the nucleoprotein of SARS virus is not adversely affected.

【0011】

The nucleoprotein of SARS virus used as the above-described immunogen

may be obtained by the following method using gene manipulation technique:

By amplifying the gene region encoding the nucleoprotein of SARS virus by PCR and cleaving the product by restriction enzyme, a DNA fragment (SEQUENCE LISTING 2) of the region encoding the above-described polypeptide substantially containing the amino acid sequence shown in SEQ ID NO:1 is obtained. Alternatively, a DNA fragment of the region encoding the above-described polypeptide substantially containing the amino acid sequence shown in SEQ ID NO:1 in SEQUENCE LISTING may be chemically synthesized based on the above-described nucleotide sequence. The thus obtained DNA fragment may be incorporated into an expression vector having an appropriate marker gene such as ampicillin-resistant gene, and a host such as *E. coli* may be transformed with the resulting recombinant DNA to obtain a transformant. By culturing the obtained transformant and by purification of the culture medium, the above-described nucleoprotein of SARS virus may be obtained.

【0012】

The above-described monoclonal antibody may be produced by a hybridoma obtained by immunizing an animal with the above-described polypeptide, and fusing anti-nucleoprotein of SARS virus antibody-producing cells obtained from the animal and tumor cells.

【0013】

The above-described hybridoma may be obtained by the following method: That is, the nucleoprotein of SARS virus obtained as described above is intrapectoneally or intravenously administered to an animal such as mouse together with Freund's complete adjuvant, dividedly in several times, at 2 to 3-week intervals, thereby immunizing the animal. Then the antigen-producing cells originated from the spleen or the like and tumor cells which can proliferate *in vitro* such as cells originated from myeloma cell line (myeloma cells) are fused.

【0014】

The above-described fusion may be attained by using polyethylene glycol in accordance with the conventional method by Kohler and Milstein (Nature, Vol.256, page 495, 1975), or by using Sendai virus or the like.

【0015】

Selection of hybridomas producing the antibody which recognizes the nucleoprotein of SARS virus from the fused cells may be attained by the following method: That is, cells which are alive in HAT medium may be selected as hybridomas from the fused cells. Then the culture medium of each of the obtained hybridomas may be reacted with highly purified nucleoprotein of SARS virus immobilized on an assay plate, and thereafter the assay plate may be reacted with anti-mouse immunoglobulin (Ig) or the like. By such an EIA, hybridomas producing monoclonal antibodies which specifically recognize the nucleoprotein of SARS virus may be selected.

【0016】

The hybridoma according to the present invention is not restricted as long as it produces a monoclonal antibody which specifically recognizes the nucleoprotein of SARS virus. Examples of the hybridoma include the 3 hybridomas established by the above-described method by the present inventors.

【0017】

The 3 hybridomas were named hybridoma rSN-18, hybridoma rSN-122 and hybridoma rSN-150, respectively.

【0018】

These hybridomas have been deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (address: 1-1-3 Higashi, Tsukuba, Ibaraki, Japan (postal code: 305-0046)). That is, hybridoma rSN-18 has been deposited under Accession No. FERMP-19572 (date of

receipt: October 24, 2003), hybridoma rSN-122 has been deposited under Accession No. FERMP-19573 (date of receipt: October 24, 2003) and hybridoma rSN-150 has been deposited under Accession No. FERMP-19574 (date of receipt: October 24, 2003).

【0019】

Each of the above-described hybridoma may be cultured in a culture medium ordinarily used for cell culture. The monoclonal antibody may be recovered from the culture supernatant. Alternatively, the above-described hybridoma may be administered to an animal of the same species as the animal from which the hybridomas were derived to accumulate ascites therein, and the monoclonal antibody may be recovered from the ascites.

【0020】

The monoclonal antibody may be recovered by a purification method conventionally employed. Examples of the purification method include gel permeation chromatography, ion-exchange chromatography and affinity chromatography using protein A.

【0021】

The reactivity of the monoclonal antibody may be confirmed by a usual method.

【0022】

The reagent for immunoassay of SARS virus according to the present invention may be produced by immobilizing the above-described monoclonal antibody on a solid phase or by labeling the above-described monoclonal antibody. As the solid phase on which the above-described monoclonal antibody is immobilized, various solid phases used in conventional immunoassays may be used. Examples of such solid phases include various solid phases such as ELISA plates, latices, gelatin particles, magnetic particles, polystyrenes and glasses, insoluble

carriers such as beads and the like. The labeled reagent may be produced by labeling an antibody with an enzyme, radioactive substance, fluorescent substance or the like. By combining these reagents, reagents used in enzyme immunoassays, radioimmunoassays, fluoroimmunoassays or the like may be produced. These measurement reagents are the reagents for measuring an antigen of interest present in the test sample by sandwich immunoassay or competitive binding immunoassay. By using the monoclonal antibody according to the present invention, an immunoassay reagent utilizing immunochromatography in which a membrane is applied as the solid phase may also be produced.

【0023】

A reagent for the above-described sandwich immunoassay may be provided by, for example, providing two monoclonal antibodies according to the present invention, and using one of them as the above-described labeled antibody and using the other as the immobilized antibody bound to the solid phase. These reagents may be reacted with a sample containing an antigen to be measured, and then the bound antigen may be reacted with the labeled monoclonal antibody (second antibody). The amount of the antigen to be measured may be determined based on the amount of the label, that is, labeled antibody, bound to the insoluble carrier, thereby attaining immunoassay.

【0024】

As a reagent for immunoassay used in competitive binding immunoassay, for example, a certain amount of a virus antigen labeled with an enzyme, radioactive substance, fluorescent substance or the like is prepared. Using this reagent, for example, a certain amount of the monoclonal antibody of the present invention, the above-described labeled virus antigen and a sample containing the antigen to be measured may be reacted competitively, and the amount of the antigen to be measured may be determined based on the amount of the labeled virus antigen bound

or not bound to the antibody, thereby attaining immunoassay. To separate the labeled antigen bound to the antibody from the one which is not bound to the antibody, the double antibody method in which immunoglobulin of the same species as the antibody and an antibody against the immunoglobulin are added and the labeled antigen bound to the antibody is precipitated to be measured, the method using charcoal or millipore filter or the like may be employed.

【0025】

In the present invention, a labeled antibody or labeled antigen may be prepared in accordance with a method such as physical adsorption or chemical bond in order to bind the above-described antibody or antigen to the solid phase or the label (see "PROTEIN, NUCLEIC ACID AND ENZYME", Extra Edition, vol.31, pp.37-45 (1987))

【0026】

The sample to be assayed by the above-described reagent is not restricted as long as it contains the nucleoprotein of SARS virus, and examples of the sample include extracts of body fluids such as nasal swab, nasal aspirate or pharyngeal swab, respiratory secretion, cell or tissue homogenates and the like, as well as serum, plasma and whole blood, originated from blood collected from human or animal. It is suspected that SARS virus infected from mammals, birds or the like to human. Thus, by measuring animal samples as well as human samples in accordance with the present invention, the infection route may be clarified.

【0027】

The nucleoprotein of SARS virus distributed in cells, tissues or the like may also be directly measured by fixing the various cells, tissues and the like originated from human or animals, and reacting the monoclonal antibody according to the present invention therewith. Further, the so called Western blotting, affinity chromatography or the like may be carried out using the monoclonal antibody

according to the present invention.

【0028】

By applying the measurement method of the nucleoprotein of SARS virus using the monoclonal antibody according to the present invention to various samples from human or animals, diagnosis of infection by SARS virus may be carried out. By using the monoclonal antibody according to the present invention, the nucleoprotein of SARS virus in various body fluids, cells, tissues and the like from human or animals may be directly measured by immunochemical or immunohistochemical method.

【Examples】

【0029】

The present invention will now be described by way of Reference Examples and Examples. However, the present invention is not restricted to the following Examples.

【0030】

Reference Example 1 Construction of Plasmid

Full length nucleoprotein gene (referred to as "*N*") consists of 1270 base pairs. Based on the reported gene sequence, oligomers of 50 to 55 bases having an overlapping of 15 bases each other were prepared. The *N* gene was divided into two fragments at the restriction site of *Nhe*I which hydrolyzes the *N* gene at about the center thereof, and each fragments was amplified sequentially by PCR. The PCR was performed by using, for the former half fragment, an end primer having an *Eco*RI site at its 5'-end region, and, for the latter half fragment, an end primer having a *Bam*HI site at its 3'-end region.

After purifying the resulting fragments by PCR Purification Kit from QIAGEN, the former half fragment was hydrolyzed with *Eco*RI and *Nhe*I, and the latter half fragment was hydrolyzed with *Nhe*I and *Bam*HI. The obtained fragments

were inserted into the *Eco*RI-*Bam*HI site of an expression plasmid pW6A shown in Fig. 1 to prepare a plasmid pWS-N. *E. coli* BL21 (DE3) (obtained from Brookhaven National Laboratory) was transformed with the obtained plasmid to obtain an ampicillin-resistant transformant *E. coli* BL21(DE3)/pWS-N. The nucleotide sequence and amino acid sequence of the nucleoprotein are shown in SEQUENCE LISTING 1 and 2, respectively.

【0031】

Reference Example 2 Expression of Recombinant Protein (S-N)

The transformant prepared in Reference Example 1 was cultured in 2 ml of LB medium containing 50 μg/ml of ampicillin at 37°C. After growing the transformant in a preliminary culture until the OD of the culture medium reached to 0.6 to 0.8, IPTG was added to a concentration of 0.4 mM to induce the expression, and the culture was continued for another 3 hours. After recovering the bacterial cells by centrifugation of 1.5 ml of culture medium at 5000 rpm for 2 minutes, the cells were suspended in 100 μl of buffer (10 mM Tris-HCl, pH8.0, 0.1 M sodium chloride, 1 mM EDTA), and the suspension was subjected to sonication for 15 minutes to completely disrupt the cells. The thus obtained product was used as the bacterial cell sample.

To 8 μl of the bacterial cell sample, 4 μl of 3 x SDS polyacrylamide buffer (0.15 M Tris-HCl, pH6.8, 6%SDS, 24% glycerol, 6mM EDTA, 2% 2-mercaptoethanol, 0.03% bromphenol blue) was added, and the resulting mixture was subjected to SDS-polyacrylamide gel electrophoresis. The sample was transferred to a nitrocellulose filter by Western blotting, and the filter was subjected to blocking with 1% BSA, followed by reacting the resulting filter with the monoclonal antibody N5 1000-fold diluted with phosphate buffer (10 mM phosphoric acid, pH7.4, containing 0.15 M sodium chloride). The resulting filter was then reacted with peroxidase-labeled anti-mouse Ig rabbit polyclonal antibody (produced by DAKO),

and, after washing, 10 ml of substrate coloring solution (0.01% aqueous hydrogen peroxide solution, 0.6 mg/ml 4-chloro-1-naphthol) was added, thereby coloring the filter. The results are shown in Fig. 2.

【0032】

Reference Example 3 Purification of Soluble S-N

The *E. coli* BL21(DE3)/pWS-N prepared in Reference Example 1 was cultured in LB medium containing ampicillin at 37°C. The transformant was grown in a preliminary culture until a cell population in terms of OD at 600 nm reached to about 0.7, and IPTG was added to 0.4 mM, thereby inducing the expression. After culturing for 18 hours, *E. coli* was recovered by centrifugation. To the recovered *E. coli*, 20 mM Tris-HCl, pH 8.0, 1 mM PMSF (phenylmethylsulfonyl fluoride) was added, and the resulting mixture was sonicated while cooling the mixture on ice. After the centrifugation, ammonium sulfate was added to the soluble fraction S-N, and 20-40% fraction was recovered. This ammonium sulfate fraction was applied to SP Sepharose First Flow (produced by AMERSHAM) equilibrated with 20 mM phosphate buffer, pH 6.9, containing 0.1 M sodium chloride and 8M urea, and eluted with 20 mM phosphate buffer, pH 6.9, containing 0.2 M sodium chloride and 8M urea, thereby carrying out purification. The eluted fraction was dialyzed against 20 mM Tris-HCl buffer, pH8.0, containing 0.2M sodium chloride. The obtained product was subjected to SDS-polyacrylamide gel electrophoresis and Western blot as in Reference Example 2, thereby confirming the degree of purification. As a result, a single band was shown.

【0033】

Example 1 Establishment of Anti-N Protein Monoclonal Antibodies

Anti-N protein monoclonal antibodies were prepared by immunizing mice with the recombinant N protein prepared in Reference Example 3, and fusing the lymphocytes from the spleen of the mice and myeloma cells. That is, BALB/C mice

were first immunized with the recombinant N protein emulsified with Freund's complete adjuvant in an amount of 50 to 100 μg/mouse, and 2 to 3 weeks later, second immunization was performed with the same antigen emulsified with Freund's incomplete adjuvant in an amount of 50 to 100 μg/mouse. The antibody titer was checked by solid phase ELISA using a 96-well ELISA plate coated with the recombinant N protein. To the mice in which the raise of the antibody titer was observed, free recombinant N protein was intravenously administered in an amount of 25 to 100 μg. Three to four days later, spleen was removed from each mouse and spleen cells were separated. The obtained spleen cells were mixed with mouse myeloma cells (P3U1) preliminarily cultured in RPMI-1640 medium at a mixing ratio of 1:2 to 1:5, and cell fusion was performed using PEG (produced by Boehringer). The fused cells were suspended in HAT medium and dividedly applied to a 96-well culture plate, followed by incubation at 37°C in a CO_2 incubator.

【0034】

The screening was carried out by the above-described solid phase ELISA. More particularly, a solution of the recombinant N protein with a concentration of 1 μg/ml was added to a 96-well ELISA plate (produced by PHARMACIA) in an amount of 50 μl/well, and the plate was left to stand overnight at 4°C, thereby adsorbing the recombinant N protein to the wells. Each well was blocked with 1% skim milk and washed three times with washing buffer (PBS containing 0.05% Tween 20). To each well, 50 μl of the supernatant of the culture medium in which cell fusion was performed was added, and the resultant was allowed to react at 37°C for 1 hour. Each well was then washed 3 times with the washing buffer in the same manner as described above, and POD-labeled anti-mouse immunoglobulin antibody (produced by DAKO) was added, followed by allowing the mixture to react at 37°C for 1 hour. After washing the wells 4 times with the washing buffer, the substrate ABTS was added, and the wells which colored were selected. The cells in the

selected wells were transferred to a 24-well culture plate and cultured in a CO_2 incubator at 37°C, and the cells were cloned by the limiting dilution method to establish 3 hybridomas which produce the anti-N protein monoclonal antibodies described below, that is, hybridomas rSN-18, rSN-122 and rSN-150. These hybridomas have been deposited with the above-described International Patent Organism Depositary under the Accession Nos. FERM P-19572, FERM P-19573 and FERM P-19574, respectively.

【0035】

Example 2 Confirmation of Reactivities of Monoclonal Antibodies by Western Blotting (WB)

The reactivity of each of the established monoclonal antibodies to the naturally occurring antigen (the N protein originated from the virus) was confirmed by WB using a concentrated virus suspension as a sample. Vero E6 cells were infected with SARS virus strain Hanoi, and the cells were cultured in a CO_2 incubator for 48 hours, followed by centrifugation of the culture medium at 2000 rpm for 15 minutes to prepare a culture supernatant ($TCID_{50}$ was 7.95×10^6/ml). The culture supernatant was inactivated at 56°C for 90 minutes, and then 31.5 ml aliquot thereof was centrifuged at 30Krpm for 3 hours using a Hitachi ultracentrifuge (40T rotor). To the obtained precipitate, TNE (Tris-NaCl-EDTA) buffer (0.3 ml) was added, and pipetting was performed to prepare a concentrated virus suspension. To this suspension, an equivolume of sample-treating solution for electrophoresis was added, and the resulting mixture was heated to obtain a test sample. After conducting SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% gel, the sample was transferred to a nitrocellulose membrane to prepare a transferred membrane for WB. After blocking the transferred membrane with skim milk, the membrane was subjected to reaction with each of the antibodies. WB was performed using as the anti-N protein monoclonal antibodies, rSN-18 antibody, rSN-

122 antibody and rSN-150 antibody, and using as a negative control, an unrelated monoclonal antibody E2CT-38 antibody.

The reaction with the antibody was performed as follows: That is, each monoclonal antibody was shaken with the antigen-transferred WB membrane at room temperature for 1 hour, thereby allowing the reaction, and the membrane was washed 3 times (washing under shaking for 5 minutes) with a washing buffer (PBS containing 0.05% Tween 20). Then a POD-labeled anti-mouse immunoglobulin antibody (produced by DAKO) was added, and the reaction was carried out for another 1 hour at room temperature. After washing 4 times (washing under shaking for 5 minutes) with the washing buffer, a substrate 4-chloronaphthol solution was added, and the bands were observed. As shown in Fig. 3, a band at a position of a molecular weight of little less than 50 Kd corresponding to the N protein was observed when each of the monoclonal antibodies was used.

【0036】

Example 3 Detection of N Protein in Virus Culture Supernatant by Sandwich ELISA

Whether an assay system for assaying the N protein may be attained or not was tested by carrying out sandwich ELISA using the recombinant N protein and virus culture supernatant. The ELISA was carried out as follows: That is, each monoclonal antibody was diluted with PBS7.4 to a concentration of 5 μg/ml, and the antibody solution was added to each well of an ELISA plate produced by FALCON in an amount of 50 μl per well, followed by leaving the ELISA plate to stand at 4°C overnight to coat the well. Then 150 μl/well of 1% BSA-PBS7.4 was added to each well, and the plate was left to stand at 37°C for 1 hour to carry out masking. Each well was washed 3 times with a washing buffer (PBS7.4 containing 0.05% Tween 20), and then the recombinant N protein and the virus culture supernatant were added to each well in an amount of 50 μl/well, followed by allowing reaction at 37°C for 1

hour. The recombinant N protein was used at a concentration of 20 ng/ml, and the culture supernatant was used as it is or after dilution with the washing buffer. The culture supernatant of the cells not infected with the virus was used as a negative control. Then each monoclonal antibody from each hybridoma culture supernatant described in Example 1 was purified by using an anti-mouse immunoglobulin affinity column and pooled, followed by labeling of the monoclonal antibody with alkaline phosphatase. The obtained labeled antibody was added to each well in an amount of 50 μl/well, and reaction was allowed to occur at 37°C for 1 hour. After washing each well 3 times with the washing buffer, the substrate *p*-nitrophenyl phosphate (p-NPP) was added in an amount of 50 μl/well, and the resulting mixture was left to stand at room temperature for 15 minutes. The wells were visually observed and absorbance at a wavelength of 405 nm was measured. As shown in Table 1, it was confirmed that detection of N protein may be attained with any of the monoclonal antibodies used in this Example.

【Table 1】

Antibody of the Present Invention	Sandwich ELISA Visual Observation		Sandwich ELISA A405	
	Virus Culture Supernatant	Control Culture Supernatant	Virus Culture Supernatant*	Recombinant N Protein
rSN-18	+	-	0.62	0.46
rSN-122	+	-	0.80	0.99
rSN-150	+	-	0.90	1.24
E2CT-38	-	-	0.05	0.10

*: used after 4-fold dilution

【Brief Description of the Drawings】

【0037】

【Fig. 1】 Fig. 1 shows a restriction map of an expression plasmid pW6A used in the present invention.

【Fig. 2】 Fig. 2 shows the results of the measurement of an expressed recombinant protein (S-N).

【Fig. 3】 Fig. 3 shows the reactivities of the monoclonal antibodies (rSN-18 antibody, rSN-122 antibody and rSN-150 antibody) confirmed by WB.

【Sequence Listing Free Text】

【0038】

5

SEQUENCE LISTING

<110> FUJIREBIO INC.

<120> Anti-SARS virus antibody

<130> P0837

<160> 2

<170> PatentIn version 3.1

<210> 1
<211> 1269
<212> DNA
<213> Coronavirus

<400> 1
atgtctgata atggacccca atcaaaccaa cgtagtgccc cccgcattac atttggtgga 60

cccacagatt caactgacaa taaccagaat ggaggacgca atggggcaag gccaaaacag 120

cgccgacccc aaggtttacc caataatact gcgtcttggt tcacagctct cactcagcat 180

ggcaaggagg aacttagatt ccctcgaggc cagggcgttc caatcaacac caatagtggt 240

ccagatgacc aaattggcta ctaccgaaga gctacccgac gagttcgtgg tggtgacggc 300

aaaatgaaag agctcagccc cagatggtac ttctattacc taggaactgg cccagaagct 360

tcacttccct acggcgctaa caaagaaggc atcgtatggg ttgcaactga gggagccttg 420

aatacaccca aagaccacat tggcacccgc aatcctaata acaatgctgc caccgtgcta 480

caacttcctc aaggaacaac attgccaaaa ggcttctacg cagagggaag cagaggcggc 540

agtcaagcct cttctcgctc ctcatcacgt agtcgcggta attcaagaaa ttcaactcct 600

ggcagcagta ggggaaattc tcctgctcga atggctagcg gaggtggtga aactgccctc 660

gcgctattgc tgctagacag attgaaccag cttgagagca aagtttctgg taaaggccaa 720

caacaacaag gccaaactgt cactaagaaa tctgctgctg aggcatctaa aaagcctcgc 780

caaaaacgta ctgccacaaa acagtacaac gtcactcaag catttgggag acgtggtcca 840

gaacaaaccc aaggaaattt cggggaccaa gacctaatca gacaaggaac tgattacaaa 900

cattggccgc aaattgcaca atttgctcca agtgcctctg cattctttgg aatgtcacgc 960

attggcatgg aagtcacacc ttcgggaaca tggctgactt atcatggagc cattaaattg 1020

gatgacaaag atccacaatt caaagacaac gtcatactgc tgaacaagca cattgacgca 1080

tacaaaacat tcccaccaac agagcctaaa aaggacaaaa agaaaaagac tgatgaagct 1140

cagcctttgc cgcagagaca aaagaagcag cccactgtga ctcttcttcc tgcggctgac 1200

atggatgatt tctccagaca acttcaaaat tccatgagtg gagcttctgc tgattcaact 1260

caggcataa 1269

<210> 2
<211> 422
<212> PRT
<213> Coronavirus

<400> 2

Met Ser Asp Asn Gly Pro Gln Ser Asn Gln Arg Ser Ala Pro Arg Ile
1 5 10 15

Thr Phe Gly Gly Pro Thr Asp Ser Thr Asp Asn Asn Gln Asn Gly Gly

```
          20                  25                  30

Arg Asn Gly Ala Arg Pro Lys Gln Arg Arg Pro Gln Gly Leu Pro Asn
        35                  40                  45

Asn Thr Ala Ser Trp Phe Thr Ala Leu Thr Gln His Gly Lys Glu Glu
    50                  55                  60

Leu Arg Phe Pro Arg Gly Gln Gly Val Pro Ile Asn Thr Asn Ser Gly
65                  70                  75                  80

Pro Asp Asp Gln Ile Gly Tyr Tyr Arg Arg Ala Thr Arg Arg Val Arg
                85                  90                  95

Gly Gly Asp Gly Lys Met Lys Glu Leu Ser Pro Arg Trp Tyr Phe Tyr
            100                 105                 110

Tyr Leu Gly Thr Gly Pro Glu Ala Ser Leu Pro Tyr Gly Ala Asn Lys
        115                 120                 125

Glu Gly Ile Val Trp Val Ala Thr Glu Gly Ala Leu Asn Thr Pro Lys
    130                 135                 140

Asp His Ile Gly Thr Arg Asn Pro Asn Asn Asn Ala Ala Thr Val Leu
145                 150                 155                 160

Gln Leu Pro Gln Gly Thr Thr Leu Pro Lys Gly Phe Tyr Ala Glu Gly
                165                 170                 175

Ser Arg Gly Gly Ser Gln Ala Ser Ser Arg Ser Ser Ser Arg Ser Arg
            180                 185                 190

Gly Asn Ser Arg Asn Ser Thr Pro Gly Ser Ser Arg Gly Asn Ser Pro
        195                 200                 205

Ala Arg Met Ala Ser Gly Gly Gly Glu Thr Ala Leu Ala Leu Leu Leu
    210                 215                 220

Leu Asp Arg Leu Asn Gln Leu Glu Ser Lys Val Ser Gly Lys Gly Gln
225                 230                 235                 240

Gln Gln Gln Gly Gln Thr Val Thr Lys Lys Ser Ala Ala Glu Ala Ser
                245                 250                 255

Lys Lys Pro Arg Gln Lys Arg Thr Ala Thr Lys Gln Tyr Asn Val Thr
            260                 265                 270

Gln Ala Phe Gly Arg Arg Gly Pro Glu Gln Thr Gln Gly Asn Phe Gly
        275                 280                 285

Asp Gln Asp Leu Ile Arg Gln Gly Thr Asp Tyr Lys His Trp Pro Gln
    290                 295                 300

Ile Ala Gln Phe Ala Pro Ser Ala Ser Ala Phe Phe Gly Met Ser Arg
305                 310                 315                 320
```

Ile Gly Met Glu Val Thr Pro Ser Gly Thr Trp Leu Thr Tyr His Gly
325 330 335

Ala Ile Lys Leu Asp Asp Lys Asp Pro Gln Phe Lys Asp Asn Val Ile
340 345 350

Leu Leu Asn Lys His Ile Asp Ala Tyr Lys Thr Phe Pro Pro Thr Glu
355 360 365

Pro Lys Lys Asp Lys Lys Lys Lys Thr Asp Glu Ala Gln Pro Leu Pro
370 375 380

Gln Arg Gln Lys Lys Gln Pro Thr Val Thr Leu Leu Pro Ala Ala Asp
385 390 395 400

Met Asp Asp Phe Ser Arg Gln Leu Gln Asn Ser Met Ser Gly Ala Ser
405 410 415

Ala Asp Ser Thr Gln Ala
420

【TITLE OF DOCUMENT】 ABSTRACT

【Abstract】

【Object】

To provide a monoclonal antibody against the nucleoprotein of the severe acute respiratory syndrome (SARS)-causing coronavirus, hybridoma which produces the monoclonal antibody and immunoassay reagent for SARS virus, which uses the monoclonal antibody as the immobilized antibody and/or labeled antibody.

【Means for Solution】

A monoclonal antibody which reacts with the nucleoprotein of SARS virus (SEQUENCE LISTING 1) obtained by using as an immunogen the nucleoprotein of said coronavirus, which nucleoprotein is expressed by a vector in which a nucleotide sequence shown in SEQUENCE LISTING 2 is incorporated, is prepared.

【Selected Drawings】 Fig. 3

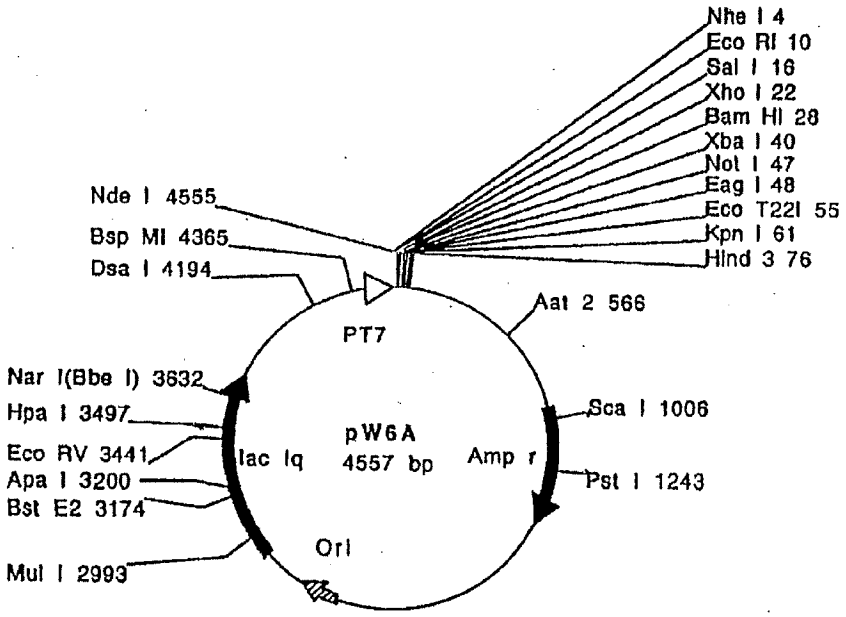
Nhe I 4
Eco RI 10
Sal I 16
Xho I 22
Bam HI 28
Xba I 40
Not I 47
Eag I 48
Eco T22I 55
Kpn I 61
Hind 3 76
Nde I 4555
Bsp MI 4365
Dsa I 4194
Aat 2 566
PT7
Nar I(Bbe I) 3632
Hpa I 3497
Eco RV 3441
Apa I 3200
Bst E2 3174
Mul I 2993
pW6A
4557 bp
lac Iq
Amp r
Sca I 1006
Pst I 1243
Ori

Fig. 1

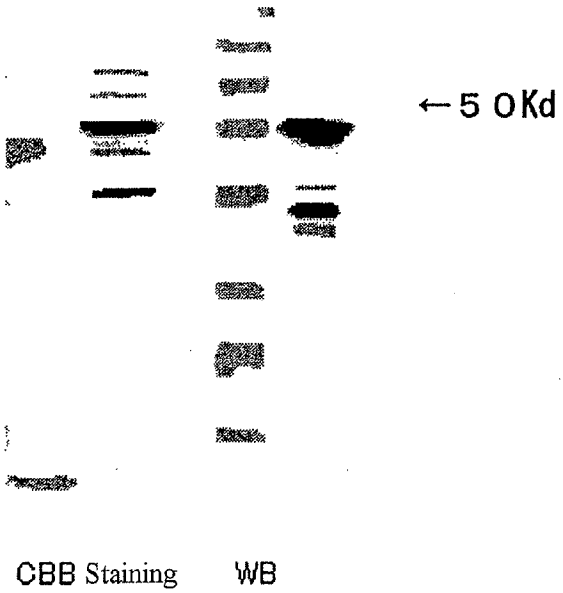
←5 0Kd
CBB Staining
WB

Fig. 2

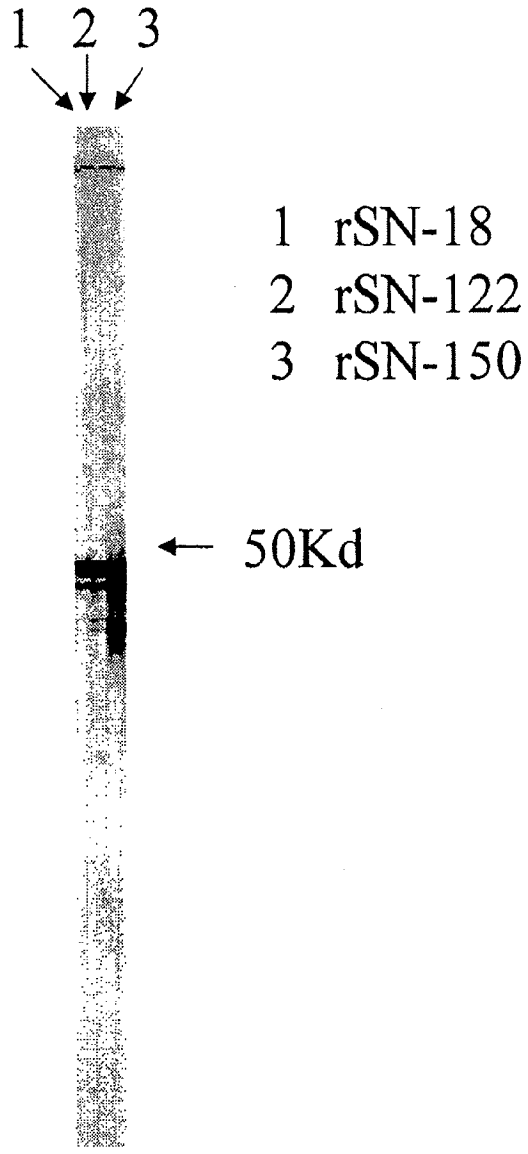

Fig. 3